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| (56)                                 | Related Art<br>KUSANO T ET AL. (1995) MOL. GEN. GENET. 248:507-517 |   |                                   |

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## ABSTRACT

This invention relates to a DNA fragment comprising a base sequence (a) the base sequence referred to as nucleotide numbers 1-3794 in a sequence number 1 in a sequence list; (b) the base sequence (a) a part of which is deleted or substituted by another base sequence, or to which another base sequence is added. Further, the invention relates to a recombinant DNA and transformed plant including the above fragment. Moreover, the invention relates to fragment developing a promoter activity with a responsive property to low temperatures which comprises a base sequence (c) the base sequence referred to as nucleotide numbers 1-2797 in a sequence number 1 in a sequence list; (d) A part of the base sequence (c) developing a promoter activity with a responsive property to low temperatures; or (e) The base sequence (c) or (d) a part of which is deleted or substituted by another base sequence, or to which another base sequence is added. Further, the invention relates to a recombinant DNA and transformed plant including the above fragment.

AUSTRALIA  
Patents Act 1990

**COMPLETE SPECIFICATION**  
**STANDARD PATENT**

**Applicant(s):**

KYUSHU UNIVERSITY

**Invention Title:**

DNA FRAGMENT, RECOMBINANT DNA, AND TRANSFORMED PLANT

The following statement is a full description of this  
invention, including the best method of performing it known to  
me/us:

- 1a -

## DNA FRAGMENT, RECOMBINANT DNA, AND TRANSFORMED PLANT

## BACKGROUND OF THE INVENTION

[0001]

## 1. Field of the Invention

This invention relates to a DNA fragment, a re-combinant DNA suitable to produce a breed such as a maize or rice having a low temperature resistance, and a transformed plant.

[0002]

## 2. Description of Related Art

From so far studies, it is found that there is a significant relationship between a low temperature resistance of a plant and a degree of unsaturation of fatty acid constructing a biomembrane thereof.

## SUMMARY OF THE INVENTION

The inventors experimentally indicated that a transformed tobacco plant comes to have a higher resistance against low temperatures when a fatty acid unsaturating enzyme gene *FAD7* derived from an arabidopsis is highly expressed in the plant.

[0003]

When a production of a certain protein in a plant cell is required, a promoter operating constitutively and having a high promoter activity has been used. Such a promoter operates continuously, which is futile in the plant.

[0004]

For instance, the constitutive promoter is also employed when a protein giving a plant a low temperature

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resistance is phenotypically expressed. Therefore, even at ordinary temperatures, the protein for giving the plant the low temperature resistance is forced to be unnecessarily expressed, causing unfavorable results.

5 For such reasons, a development of a site-specific and inducible promoter has been required. Particularly, it is required that an expression of a specific gene is strengthened only at low temperatures, in order to produce a breeding intermediate mother body in  
10 which a gene expressing an enzyme unsaturating a fatty acid and other protein genes contributing to a low temperature resistance is inducibly expressed in response to low temperatures, or in order to make possible production of an unstable functional protein using a plant cell.

15 The invention provides a promoter capable of strengthening expression of a fatty-acid-unsaturating-enzyme gene and other protein genes contributing to a low temperature resistance in response to low temperatures.

20 The invention further provides a breed having a low temperature resistance using the above promoter.

The invention also provides a means of utilizing the promoter in an inducibly generating system of a functional protein in a plant cell being specific to low temperatures.

25 According to an aspect of the invention, there is the provision of a recombinant DNA and transformed plant having a low temperature resistance and induced a recombinant DNA including the DNA fragment mentioned above, respectively.

30 According to a second aspect of the invention, there is the provision of a recombinant DNA which includes the DNA fragment comprising the base sequence (c), (d), or (e) described above, and a transformed plant which transduces the recombinant DNA and phenotypically expresses  
35 a specific protein in response to low temperatures.

Since a rice's low temperature inducible gene lip19 or maize's low temperature inducible gene mlip15



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codes a DNA binding factor, they are considered to be a gene controlling transcription of other gene groups induced or repressed under low temperature stress. The inventors have clarified a functional unit of a low temperature inducible promoter in the maize's mlip15 gene and that the functional unit also operates in a transformed plant.

The inventors has isolated a genomic clone of the maize's mlip15 gene by an common method. Due to determination of nucleotide sequence, it becomes clear that the gene does not include a intron (see: Sequence number 1 in Sequence list).

In the gene mlip15, 2.8 kb of a nucleotide sequence (0.6 kb of a nontranslated region at 5'-end and 2.2 kb of a nucleotide sequence linking at upstream thereof, in a mlip15 cDNA) and 2.2 kb of a nucleotide sequence (the nucleotide sequence remained after subtracting 0.6 kb of the non-translated region at 5'-end from 2.8 kb of the nucleotide sequence) are respectively bound with a  $\beta$ -glucuronidase gene as a reporter gene to make recombinant DNAs.

Each recombinant DNA is introduced into a callus derived from a rice's scutellum by using a particle gun.

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The result is that the former maintains a reactivity for low temperatures but the latter lose it. Therefore, it has been clarified that the 2.8 kb fragment containing 0.6 kb of the nontranslated region at 5'-end has a promoter function responsive to low temperatures.

[0017]

In the invention, as a vector used for producing a recombinant DNA, a plasmid can, for example, be employed. Further, as a plant used for introducing the recombinant DNA, a useful cultivating monocotyledon such as maize, rice, wheat, barley, oat, Italian millet, or Japanese millet is preferably used.

A protein produced by induction of the promoter according to the invention includes an  $\omega$ -3-fatty acid unsaturating enzyme etc.

Moreover, the invention includes a base sequence one or several nucleotides of which are deleted or substituted by another base sequence, or to which another base sequence is added, if it maintains the promoter function according to the invention.

## BRIEF DESCRIPTION OF THE DRAWING

The invention will be described with reference to the accompanying drawing, wherein:

Fig. 1 is an illustration diagrammatically showing *mlip15* region.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0018]

(Isolation of *mlip15* genomic clone and determination of the



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nucleotide sequence)

A genome DNA prepared from a maize (breed: honey bantam) is cut (partially digested) by a restriction enzyme Sau3A and then separated by using a sucrose density gradient centrifugation to obtain 9.7-22 kb of DNA fraction. The DNA fraction is bound with a  $\lambda$ -EMBL3 digested by BamHI and then makes phage particles by using Giga-packGoldIIkit.

By examining them by using Escherichia coli XL1-Blue MRA (P2) as a host,  $1 \times 10^7$  pfu/ml of a library is obtained. The library is selected by using the whole length of the mlipl5 cDNA as a probe to obtain three positive clones.

Among them, a clone named " $\lambda$ H1" is found to include 11.5 kb of a fragment containing the mlipl5 cDNA, 5 kb of a fragment at the 5'-end, upstream thereof, and 5 kb of fragment at the 3'-end, downstream thereof.

[0019]

In Sequence list 1 is shown 3,794 bp of a base sequence being an EcoRI-BamHI fragment including (consisting of) the whole length of the mlipl5 cDNA.

A putative amino acid sequence at the region coding an mlipl5 protein is symbolized under the base sequence by one capital letter. Nucleotide numbers corresponding to the assumed amino acid sequence are 2798-3205. A terminal codon of the base sequence is shown as a mark "\*\*\*". A nucleotide sequence which is assumed as "TATA box" is underlined and also has a description of "TATA box" thereunder. A transcription initiation point of mlipl5 is T as nucleotide number 2272.

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[0020]

(Constructing of recombinant DNA used for introducing to rice callus)

A fragment having the nucleotide numbers 1-2797 and  
5 a fragment having the nucleotide numbers 1-2271 are  
respectively amplified by a PCR method (a polymerase chain  
reaction method) using the maize'mlip15 genomic clone as a  
template. The fragment of the nucleotide numbers 1-2797 is  
the above mentioned genomic sequence of 2.8 kb which consists  
10 of 0.6 kb of the nontranslated region at 5'-end and 2.2 kb of  
genomic sequence at upstream of the 0.6 kb of the non-  
translated region at 5'-end, in mlip15 cDNA . The fragment  
of the nucleotide numbers 1-2271 is the above mentioned  
genomic sequence of 2.2 kb and consists of the rest of 2.8 kb  
15 of the nucleotide sequence subtracted by 0.6 kb of the  
nontranslated region at 5'-end.

A nucleotide numbers 2798-3205 is a region coding  
the mlip15 protein.

[0021]

20 As an enzyme in the above amplification, LATaqDNA  
polymerase having a proofreading activity is used. In this  
case, each primer is such designed that the base sequence of  
the nucleotide numbers 1-6 becomes a Hind III site (AAGCTT)  
and the base sequence of the nucleotide numbers 2792-2797 and  
25 the base sequence of the nucleotide numbers 2266-2271 respec-  
tively become a Bam HI site (GGATCC) to obtain each primer.

[0022]

Each amplified fragment is integrated between the

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Bam HI and Hind III sites of a vector used for an *Escherichia coli* pUC18 and a confirmation of each base sequence is conducted. Using each plasmid with each base sequence confirmed, each Bam HI - Hind III fragment respectively  
5 consisting of 2797 bp and 2271 bp is reproduced and integrated between the Bam HI and Hind III sites of pBI 221. Each obtained recombinant plasmid is respectively named pBImp28 (because of containing 2.8 kb of promoter region in the mlipl5) and pBImp22 (because of containing 2.2 kb of  
10 promoter region in the mlipl5).  
[0023]

In Figure 1, the mlipl5 promoter region is diagrammatically illustrated. Beside, the mlipl5 promoter portion contained in the above recombinant plasmid is shown therein.  
15 The symbol of "+1" indicates a transcription initiation point (the nucleotide number 2272), the symbol of "key arrow" the direction of transcription, and the symbol of "ATG" the position of a transcription initiation codon (the nucleotide number 2798). A  $\beta$ -glucuronidase gene (abbreviated as GUS)  
20 is used as a reporter gene (its scale is not correct in the figure).  
[0024]

(Reactivity of the introduced plasmid in low temperatures)

To a callus derived from a rice' scutellum (breed:  
25 Notohikari), the above each plasmid is introduced using a particle-gun (made by BIO-RAD Co., Ltd.) After the introduction, the callus is incubated for 24 hours in a dark place at 25°C and then divided to two parts homogeneously. After

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respectively incubating for another 24 hours one at 25°C and the other at 5°C, a GUS test is carried therefor. A GUS activity is judged as whether a low temperature reactivity is present or not, by calculating a ratio of the number of blue spots per scutellum on an X-Gluc as substrate at 5°C to that of 25°C (see: Table 1). Three times of tests are repeated on both plasmids and the results are shown in Table 1.

[0025]

Table 1

| Induced plasmid | Responsive property to low temperatures |      |
|-----------------|---|------|
|                 | Ratio of GUS activity(5°C/25°C)         |      |
| pBImp28         | Experimental 1                          | 2.7  |
|                 | Experimental 2                          | 6.0  |
|                 | Experimental 3                          | 4.7  |
| pBImp22         | Experimental 1                          | 1.1  |
|                 | Experimental 2                          | 0.62 |
|                 | Experimental 3                          | 0.57 |

[0026]

10 When the pBImp28 is introduced, a high GUS activity is obtained. But, when the pBImp22 is introduced, the GUS activity is inferior thereto. The following may be derived from these results.

- 15 (a) In DNA fragment of the nucleotide numbers 1-3794 of the maize' mlip15 genomic clone, the promoter region relating to the low temperature responsive property exists at upstream of the putative amino acid sequence coding the mlip15 protein.
- (b) The fragment of the nucleotide numbers 1-2797 contains the promoter region relating to the low temperature

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responsive property.

(c) The fragment of the nucleotide numbers 1-2271 is expected to effect as the promoter, but the fragment itself has a low responsive property to low temperatures. The fragment itself of the nucleotide numbers 2272-2797 has the low temperature responsive property and relates to the expression of the promoter.

[0027]

Sequence list

10 Applicant name : President of Kyushu university  
 Title of invention : DNA fragment, recombinant DNA, and transformed plant  
 Number of sequence : 1  
 Sequence number : 1  
 15 Sequence length : 3794  
 Sequence type : Nucleic acid  
 Number of strand : Double strands  
 Topology : Linear  
 Original source  
 20 Organism : Maize breed of honey bantam  
 Genome of mlip15 gene

Sequence: Genomic sequence of mlip15

1 GAATTCGAATAACGGCCCCGCATGCAACCAGATAGCGGATCTTCGGCGCTAAACTCA 60  
 61 GAGGGAAGCAATTGCCGAAGAGTCGGCGTGCAAGAATAACATAAGTAGATAAGATTTCA 120  
 121 CGATCTATAAAAGGATATCTCCCTAGTCGGCTATATAAGGCTAGGAGGTACCCAAACAA 180  
 181 AACGAATCACTCTCTTCAACCATAACGCCCACTAGTAGACTAATGAGATCTCATC 240  
 241 CACCGTCACCCGGGAATCATCTGTAACCCAAGCAAACCTCAATACCCAACATCACACATGA 300  
 301 CTTAGGGTATTACGCATTTAGCGACCGAACCTGTATAATTTCTTGTTTCAACGTG 360

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361 CACCTGCACGTACCATCGAGTTGCGATTAACTGCGCGTCTCCAAAAAATACTGGTGGT 420  
421 CCTCCCAAAAACTCGACCGGACGATAGAAATAGTGTATGGCTAAGTAGAGCAAGGTGCGG 480  
481 TTTGGTCCGGGCTATGATGAGAATCCAGTAGACTGAATCCACGTGCTAAACCAACATC 540  
541 ACTGGTTTGGCTGCATCTTCTCGGATTACGTGTGTTGTGCAAATTCCTATAATCCACGG 600  
601 CAACCAGACGGGGGCGCAACCAATTGGTGTTCCTAGGAAGCCCCCGGTTACATTGGAT 660  
661 CATAGGATGATTCAAGGGTTATGATTTTTAGCTACTAATTGGTGTGCATCATGGTTTAT 720  
721 AGGTGAAGATTGTTATTCAATCAAAGGGCGACATATCCCTCCGCGTTTAGAGACTTGCGT 780  
781 GTAGTGTAACATGGATGTAATTGTGCTACCTTTAATAGAGTCCCTTAGCTCTTCAAAA 840  
841 CAAATCTTATTATATTAATTACTAGTCCATCCATTTTATTCTAATTTAGTTTCGAAAT 900  
901 TACTAAATATAGAAAATAAATAGAGTTTTAGTAGCAATATGAAAAGTAAATATAGTT 960  
961 TTAATTTCCGTATTTAGTGATTTAATACTAAAATAATAAAATGGAGAGACTAAAAAC 1020  
1021 TAGTCCCTATAAACCAACATTCCTTAAATAAGCCCCGTTGGCTAGGACAATGACCTATT 1080  
1081 TTTTCTCGCAACCGGAAGAATAAAAAATTCACCGTAACCTTCTTTCTTTCTTTT 1140  
1141 TGCGAAAGAAGATAGTTGCAAGACGAATCCAGAGTTTATCTGGAAGAAGAAAGTTCCTAA 1200  
1201 TCCTCCTCCTTCCCTGTAGATATTATCAGCAAGGCAAGCGTGTACGGCTTCTTGCTGA 1260  
1261 GTAATCCGCTCCTATTTTTTTTTTGGGAGGGCGCTTCCTACCGCTTCGCTTCTAAAC 1320  
1321 GGTGGGCAAAATTTGGTACGATAAAGAAAAAGAGGAGGACGAGTGGGAGGGCACTTCTGG 1380  
1381 AAAAACTTTTTAATGAGCTGGACCAAGCAGCTGGGCAAGCTGTCACTAGGACTGGACAA 1440  
1441 AATACTCGTGGCTGATAACTCGCTCGACTCGGCTCGTTAGTAGCTCAGCTCGACTCGGC 1500  
1501 TCGTTTAATTTGTAGCGAGCCAAGCTAGCATTCTAGCTCGATTCTCTAATGAGCCAGC 1560  
1561 TCGGTTAGCTCGTGAGCTAGCTCGGAGCCAAACGAGCTAAGCCACAACACAAATTTGT 1620  
1621 CTAGTCATTGATGTGCTCATCTCTCATAGTCTTGTTTTCTCGTAGTTATGATCTGTGA 1680  
1681 TATGGACATGTGCGATGTGCCATGTGCTTAAATATTTATATTGTCATGGCTACATGT 1740  
1741 TTGTAGTGTTAAATACTTAAATATAATTTTCGGTTATAAATATTTATGTACATAGA 1800  
1801 TATTTATATTTAGTTGTGTGCTCAGGAGCTAAGAGCTGGCTCGAGCTTCCTAACGAG 1860  
1861 CCGAGCCGAGCCAGCTATTTAGCTCGTTAGTATAACGAGCCGAGCCGAGCTGGCTCGTTA 1920  
1921 TAGTAACGAGCCATAACGAGCCGAGCCATAACGAGCCAAGCTGGTTCGATATCCACCCCT 1980  
1981 AGCTGTACCGTCCGCCAGTCCGCTTCGTTCCGTCAGCGGGCCACCTCATCTGCATT 2040

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2041 TTCCATTCTCGTCTCCGACCTCATCTGCAATTTCCAGCCAAGTAGTAGTAACTAGT 2100  
2101 GCGCGTCCCGTGGCGTGGCATCAGGAAAAGAATATGCCGTCCAGCCACCATCCCCC 2160  
2161 ACCGTCCCGAAATCCAGAACTACCCTCGGCTCCAGCTATAAATAGCCGCCCGGGAGA 2220

putative TATA box

2221 CGTTCGAAACCTTCCCATCTCCGATAAAAGATAAGGAGTGTCTCTCTCTTTTCAGC 2280

cDNA start site

2281 TAAGTCCCTGCTCCCTCTCTTTTCTTACATTAGGTCTCGCAGCTCCTCTCTTTTTC 2340  
2341 TTGTTTCTTTCTTTGATCTGCGAGCCGTCCAGTCCAGTACTCTCTTTCCGTGAAGGA 2400  
2401 ACTCTTGACGCCGCCCTCTGGTTTCTCGAATTCTTGTTCCCGGTCCCTCCTCTGT 2460  
2461 CCCCGGTAGATCCGTCCGTCCGAGGACACACGTCCCACCCCATGTTTACCCACCA 2520  
2521 GTTCTCTGACGGCGCGCTGCTCCGATGAAGCTGAGCGTCTCGTATCCGCGCTCC 2580  
2581 ACTCCTTCTCCGTGCCCTTCTCTACTGTTCTACGTCTTCTCATGAACGCATCGCCCT 2640  
2641 CTCACCTGCTGATCCTTCCGCATCTCTCCATCTCTTTCTCTGAGATAGCTTTCCG 2700  
2701 AATCCATCTCTAGGGCTCTTGTTTCTCCCATCTCCCCCACCACCCACCCACCAAC 2760  
2761 ACAAGTCCCCTTGTTCAATCCGACAAGACAAGCATCCATGTCGTGTCACGCCGAGCTC 2820

M S S S R R S S

2821 GAGCCCCGACAGCAACGACACGACGAGCGAGCGCAAGCGGAAGCGGATGCTGTCCAACAG 2880

S P D S N D T T D E R K R K R M L S N R

2881 GGAGTCCGCCCGCGGTCCGCCCGCGGAAGCAGCAGCGGCTGGAGGAGCTGGTGGCGGA 2940

E S A R R S R A R K Q Q R L E E L V A E

2941 GGTGCCCGCCTGCAGCGGAGAACGCGGCGACGAGGCCCGCACCGCGCGCTGGAGCG 3000

V A R L Q A E N A A T Q A R T A A L E R

3001 CGACCTGGCAGGCTGACGGGACAAACCGGTCTGCGCGCCGCCACGCGAGCTGGC 3060

D L G R V D G D N A V V R A R H A E L A

3061 CGGCCGCTGAGTCTGCGCGCGGTCTCGAGTGCTCCAGATGGCCGGCGCGCGT 3120

G R L Q S L G G V L E V L Q M A G A A V

3121 CGACATCCCGAGATGTCACCGACGACCCATGCTCCGCCCTGGCAGCGCTCTTCCC 3180

D I P E M V T D D P M L R P W Q P S F P

- 14 -

3181 CCCGATGCAGCCCATCGGTTCTGAGAATCTGAGDCTCAGCCGGCGGAGAGGCCAATT 3240

P M Q P I G F \*

3241 TCTGTGTCGTGCGGCTGTCTATCTCGTATTGGTATATCTATTCAAAATCATCCTTGTC 3300

3301 ATGGTTTGCTCTTCTTGTTCAGTGTATAAATTTGCTTCTTGTAGTGTATAAATTTGG 3360

3361 CCATCGGAAGGATGTGTTGTAGTGTAAATATCTTGTGGAGTGTAAATATCTTATCT 3420

3421 TGCTTATGAAATCGAATATGCCTATATATATGTTATGCTGTACGAGTATGGGCTCCA 3480

polyA additional site

3481 AATTGTGAGCCTTCTGTCTGTTATGGTGAGGCGATGAATCCAATTTGTGAGCACACATG 3540

3541 AATCAATTCGAGATTCGACATGTCAAGTTGATCGTTGCAGGAAGGACGGTTTTTGTATG 3600

3601 GACGGACATACCAAGTTACTGCATTTTACTTAAATATCTCACTTATTTTTAGATCGGC 3660

3661 ATTTCTCCACTCGTTAGATTTCTTGTCTTGAGTCAGAAGATACTACAGCATGTCATAT 3720

3721 CTCAATTGGAATACCATTAGGGTCCCTCATCTTAACCTATTTTCATCTCTTTAATACGTA 3780

3781 GATTTTTTGGATCC 3794

In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprising" is used in the sense of "including", i.e. the features specified may be associated with further features in various embodiments of the invention.



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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A DNA molecule comprising the nucleotide sequence 1 to 3794 shown in sequence 1, or a functional fragment thereof, wherein said DNA codes for a promoter which is induced by low temperature or a structural gene coding for mlip15 protein.
2. A DNA molecule comprising the nucleotide sequence 1 to 2797 shown in sequence 1, or a functional fragment thereof, wherein said DNA codes for a promoter which is induced by low temperature.
3. A DNA molecule comprising the nucleotide sequence 1 to 2271 shown in sequence 1, or a functional fragment thereof, wherein said DNA codes for a promoter which is induced by low temperature.
4. A DNA molecule which has at least 75% sequence homology with a DNA according to any one of claims 1 to 3.
5. A DNA molecule which has at least 85% sequence homology with a DNA according to any one of claims 1 to 3.
6. A DNA molecule which has at least 95% sequence homology with a DNA according to any one of claims 1 to 3.
7. A recombinant DNA molecule comprising a DNA molecule according to any one of claims 1 to 6.
8. A transformed plant having a low temperature resistance, wherein said plant comprises a DNA molecule according to any one of claims 1 to 7.
9. A transformed plant phenotypically expressing a specific protein in response to low temperature, wherein said plant comprises a DNA molecule according to any one of claims 1 to 7.
10. A DNA according to claim 1 substantially as hereinbefore described with reference to any of the examples.

Dated this 25<sup>th</sup> day of June 1999

35 KYUSHU UNIVERSITY

By their Patent Attorneys

GRIFFITH HACK

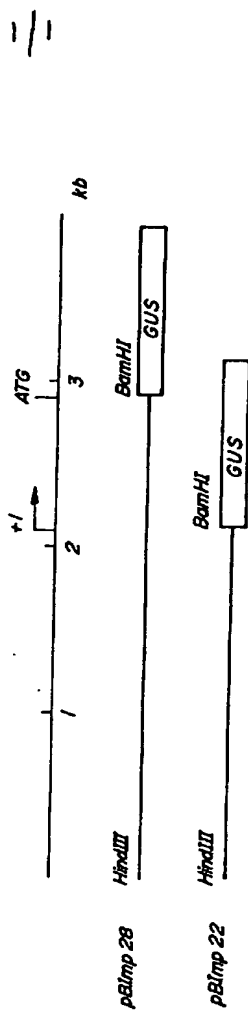
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FIG. 1



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